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Abstract: Early diagnosis of endometritis in dairy cattle is currently requires invasive techniques and specialist expertise. The goal of this study is to utilize a gel-free mass-spectrometry based proteomics approach to compare the plasma proteome of dairy cattle with cytological endometritis to those without. Blood samples were collected from cows (N = 112) seven days postpartum (DPP). Plasma samples from a cohort of 20 animals with cytological endometritis (n = 10) and without (n = 10) as classified 21 DPP were selected for proteomic analysis. Differential abundances of proteins between the two animal groups were determined using both fold change (1.5 fold change) and statistical significance threshold ($p < .05$). A total of 181 non-redundant proteins were quantified, and 25 proteins were found with differential abundance. These include 4 binding protein alpha and mannose binding lectin 2 involved in immune responses. Differentially abundant proteins between the animals were then processed using PANTHER for gene ontology. Gene ontology included associations with innate immune processes, acute phase responses and immune regulation. A potential marker for disease identified here is the "uncharacterized protein G5E513," a protein previously defined by RNA-transcripts. These proteins may form the basis for endometritis prognosis, the development of which is proceeded by systemic changes in immune function. SIGNIFICANCE: Endometritis is a costly reproductive disease of lactating dairy cows that warrants timely diagnosis. We utilized a gel-free mass-spectrometry based proteomics approach to compare the plasma proteome of dairy cattle with cytological endometritis to those without, for the characterization of changes in the proteomic profile associated with uterine disease postpartum. Furthermore, we compared the plasma proteome of healthy and affected cows in the same physiological status of production to better understand the relationship between changes in expression of circulating proteins and to unravel essential biological mechanisms involved in bovine cytological endometritis.

DOI: https://doi.org/10.1016/j.jprot.2019.103421

Miller, Blake A; Brewer, Amy; Nanni, Paolo; Lim, Joseph J; Callanan, John J; Grossmann, Jonas; Kunz, Laura; de Almeida, André M; Meade, Kieran G; Chapwanya, Aspinas (2019). Characterization of circulating plasma proteins in dairy cows with cytological endometritis. Journal of Proteomics, 205:103421. DOI: https://doi.org/10.1016/j.jprot.2019.103421

Journal of Proteomics 205 (2019) 103421

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Characterization of circulating plasma proteins in dairy cows with cytological endometritis

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A B S T R A C T

Early diagnosis of endometritis in dairy cattle is currently requires invasive techniques and specialist expertise. The goal of this study is to utilize a gel-free mass-spectrometry based proteomics approach to compare the plasma proteome of dairy cattle with cytological endometritis to those without. Blood samples were collected from cows ($N = 112$) seven days postpartum (DPP). Plasma samples from a cohort of 20 animals with cytological endometritis ($n = 10$) and without ($n = 10$) as classified 21 DPP were selected for proteomic analysis. Differential abundances of proteins between the two animal groups were determined using both fold change (≥ 1.5 fold change) and statistical significance threshold ($p < .05$). A total of 181 non-redundant proteins were quantified, and 25 proteins were found with differential abundance. These include 4 binding protein alpha and mannose binding lectin 2 involved in immune responses. Differentially abundant proteins between the animals were then processed using PANTHER for gene ontology. Gene ontology included associations with innate immune processes, acute phase responses and immune regulation. A potential marker for disease identified here is the "uncharacterized protein G5E513," a protein previously defined by RNA-transcripts.

These proteins may form the basis for endometritis prognosis, the development of which is proceeded by systemic changes in immune function.

Significance: Endometritis is a costly reproductive disease of lactating dairy cows that warrants timely diagnosis. We utilized a gel-free mass-spectrometry based proteomics approach to compare the plasma proteome of dairy cattle with cytological endometritis to those without, for the characterization of changes in the proteomic profile associated with uterine disease postpartum. Furthermore, we compared the plasma proteome of healthy and affected cows in the same physiological status of production to better understand the relationship between changes in expression of circulating proteins and to unravel essential biological mechanisms involved in bovine cytological endometritis.

1. Introduction

Endometritis affects cattle postpartum, and decreases dairy industry profitability [1]. Dairy cattle have been the subject of intense genetic selection for milk production over the last 50 years resulting in susceptibility to uterine disease and decreased fertility [2]. After calving, the uterus undergoes important anatomical, physiological and immunological changes necessary for bacteria clearance, physiological inflammation and uterine involution [3,4]. These endometrial morphological and secretory changes reflect the extremely complex interactions between RNA and protein. Usually, cows are able to clear bacteria from the uterus and resolve inflammation before the third week postpartum. Inappropriate activation of the immune response early postpartum perturbs tissue remodeling, delays bacterial clearance and prolongs endometrial inflammation [5]. This results in uterine disease and often decreased milk yield [1,6]. Currently, diagnoses for endometritis are performed 21 days postpartum (DPP) using clinical assessment, metricheck instrument or cytological evaluation [7,8]. However, these methods of disease detection are not practical for regular on-farm use and therefore early postpartum biomarkers would be beneficial. In addition, previous studies have identified transcriptomic differences both locally [9] between cows that resolve inflammation and those that develop endometritis as early as 7 DPP, but it is not known whether these differences also exist at the protein level. Because it is often difficult to predict the function of a protein based on homology to other proteins or genes encoding them, this proteomic analysis provide insight into comparison of protein levels with potential application for this economically important endometrial diseases [10]. Therefore, proteomic analyses are powerful tools for the identification of proteins differentially expressed, more so in the endometria of cattle with endometritis.

However, the plasma protein profiles of cattle cytological

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https://doi.org/10.1016/j.jprot.2019.103421
Received 11 February 2019; Received in revised form 27 May 2019
Available online 22 June 2019

endometritis are not fully understood, and there are still many unknown potential disease-associated proteins. The comparison of relative protein abundance between animals postpartum would identify useful proteins that could be used to distinguish healthy cattle from those with a pathological inflammation. To that end, we propose to use a label-free liquid chromatography mass spectrometry (LC-MS) proteomics approach to compare the blood plasma proteomes of postpartum dairy cattle with and without endometritis. Because plasma is an amorphous component of blood, differential abundances of plasma proteins can distinguish physiological or pathological status of mammals including cattle [4,11,12]. Therefore, plasma is an ideal medium to focus research for elucidating disease-associated factors for diagnosing endometritis. Detectable proteomic changes in the peripheral blood of an animal will become a convenient tool for describing changes that are prognostic of immune system disorders in cattle that develop endometritis. We previously confirmed that using gene expression analysis is a viable way to detect endometritis at 7 DPP (Lim et al., 2018 unpublished). Upregulation of innate immune genes such as interleukin 6 (IL6), IL8, tumour necrosis factor alpha (TNFA) expression was reported in animals with cytological endometritis [13]. Correlations between gene expression and relative protein abundance is low, therefore it is important to take both transcriptomic and proteomic data into consideration [14,15].

Since proteins represents the final effective molecule entity within an organism and are the primary interaction between genome and environment, the aim of the present study is to describe the proteomic profiles of cattle with and without endometritis, and identify possible biomarkers that may predict at risk animals early postpartum.

2. Materials and methods

2.1. Ethics statement

All procedures described were conducted under ethical approval and experimental license from the Irish Health Products Regulatory Authority in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendments of the Cruelty to Animals Act 1876) Regulations, 1994.

2.2. Animal management, blood sampling and total protein measurement

Sample collection was performed on a commercial, spring-calving dairy farm in Ireland with an average milk yield of 6417 l /year. The animals (3–5 years old, second to third parity) were Holstein-Friesian cows calved indoors. Animals that experienced dystocia (calving assistance) were not included in the study. Blood samples (9 mL) were collected (by coccygeal venipuncture) from Holstein dairy cows 7 days postpartum (DPP) ($n = 112$) using vacutainer tubes with Lithium Heparin anticoagulant. All the animals were in the same physiological status. Blood was brought back to the laboratory within 3 h and centrifuged at 2000 x*g* for 15 min at 4 °C to collect the plasma. Following cytological evaluation performed at 21 DPP, 20 animals were retrospectively selected for proteomic analysis in this study. This included 10 healthy cows and 10 with cytological endometritis (Table 1). These groups were further subdivided based on whether the endometrial inflammation was prominent or low-grade at 7 and 21 DPP. Clinical history (breeding dates, parity, milk yield) was recorded for each cow. All plasma samples were stored at −80 °C until further analysis.

2.3. Cytological classification

Endometrial cytobrushes were collected at 7 and 21 DPP using double guarded sheaths to avoid contamination. The brush was guided trans-rectally to ensure that it was well positioned. Once the cervix was reached, the inner brush was pushed through the outer guard and rotated 3 times against the uterine mucosa to collect endometrial cells. The brush was removed and rolled anti-clockwise on a glass microscope slide [16]. Slides were air-dried, fixed and stained with Diff-Quick (a modified Wright-Giemsa® stain) as previously described [17]. Cytological assessment was performed by counting the number of polymorphonuclear (PMN) cells and epithelial cells present [17]. A total of 200 cells were counted at 400× magnification, using 10 separate fields of view. Animals with > 18% PMN 21 DPP were diagnosed with cytological endometritis in line with recent publications [17,18]. Those with cell counts under 18% were considered healthy. At 7 DPP, all animals are expected to have significant inflammatory cell numbers as part of the early changes within the uterus postpartum and thus theses samples were also grouped based on their inflammatory status. This was defined as high (H) inflammation if the sample contained > 18% PMN or low (L) inflammation if below 18%. The letters H and L were also applied to the samples 21 DPP with H denoting endometritis and L denoting healthy. With those two time points, it was therefore possible to divide the 20 animals into four categories HL, HH, LL and LH (displaying endometrial status as shown in Table 1, each category contained five individual animals).

2.4. Sample preparation for protein quantification

The protein concentration was estimated for each plasma sample using the Qubit® Protein Assay Kit (Life Technologies, Zurich, Switzerland). The samples were then prepared by using a commercial iST Kit (PreOmics, Plannegg, Germany) with an adapted version of the protocol. Briefly, 50 µg of protein were solubilized in 'Lyse' buffer, boiled at 95 °C for 10 min and processed with High Intensity Focused Ultrasound (HIFU) for 30s setting the ultrasonic amplitude to 85%. Then the samples were transferred to the cartridge and digested by adding 50 µl of the 'Digest' solution. After 60 min of incubation at 37 °C the digestion was stopped with 100 µl of Stop solution. The solutions in the cartridge were removed by centrifugation at 3800 ×*g*, while the peptides were retained by the iST-filter. Finally, the peptides were washed, eluted, dried and re-solubilized in 'LC-Load' buffer for MS-Analysis.

2.5. Liquid chromatography-mass spectrometry analysis

Mass spectrometry analysis was performed on a QExactive mass spectrometer coupled to a Nano EasyLC 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. For each sample 4 µL of peptides were loaded on a commercial Acclaim PepMapTM Trap Column (75 µm × 20 mm, Thermo

Table 1
Animal IDs of the 20 animals and their neutrophil counts (high (H) or low (L)) are shown for 7 (column 2) and 21 days postpartum (column 3). The animals were divided into four categories (HL, HH, LL, LH), each catagory containing 5 animals. The fourth and final column lists whether animals were healthy or had cytological endometritis at the end of the study period.

Animal IDs	Neutrophil count (7 DPP)	Neutrophil count (21 DPP)	Health status of animals at 21 DPP
1–5	High	Low	Healthy
6–10	High	High	Cytological endometritis
11–15	Low	Low	Healthy
16–20	Low	High	Cytological endometritis

Fisher Scientific, Waltham, MA, USA) followed by a PepMapTM RSLC C18 Snail Column (75 μm × 500 mm, Thermo Fisher Scientific, Waltham, MA, USA). The peptides were eluted at a flow rate of 300 nL/min by a gradient from 3 to 30% B in 95 min, 47% B in 4 min and 98% B in 4 min. All 20 samples were acquired in a randomized order without technical replication. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectrum (300–1700 *m/z*) at a resolution of 70,000 at 200 m/z after accumulation to a target value of 3,000,000, followed by HCD (higher-energy collision dissociation) fragmentation on the twelve most intense signals per cycle. HCD spectra were acquired at a resolution of 35,000 using a normalized collision energy of 25 and a maximum injection time of 120 ms. The automatic gain control (AGC) was set to 50,000 ions. Charge state screening was enabled and singly and unassigned charge states were rejected. Only precursors with intensity above 8300 were selected for MS/MS (2% underfill ratio). Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on *m/z* 371.1010 and 445.1200.

2.6. Protein identification and label-free quantification

The acquired raw MS data were processed by MaxQuant (version 1.4.1.2) [19], followed by protein identification using the integrated Andromeda search engine. Each file was kept separate in the experimental design to obtain individual quantitative values. Spectra were searched against the uniprot *Bos taurus* (taxonomy 9913) reference proteome (canonical version from 2017 to 08-17, 24,233 forward sequences), concatenated to its reversed decoyed fasta database and common protein contaminants. Carbamidomethylation of cysteine was set as fixed modification, while methionine oxidation and N-terminal protein acetylation were set as variable. Enzyme specificity was set to trypsin/P allowing a minimal peptide length of 7 amino acids and a maximum of two missed-cleavages. Precursor and fragment tolerance were set to 10 ppm and 20 ppm, respectively for the initial search. The maximum false discovery rate (FDR) was set to 0.01 for peptides and 0.05 for proteins. Label free quantification was enabled and a 2 min window for match between runs was applied. The re-quantify option was selected. For protein abundance the intensity (Intensity) as expressed in the protein groups file was used, corresponding to the sum of the precursor intensities of all identified peptides for the respective protein group. Only quantifiable proteins (defined as protein groups showing two or more razor peptides) were considered for subsequent analyses. Protein abundance data were transformed (hyperbolic arcsine transformation) and missing values (zeros) were imputed using the missForest R-package, package version 1.4. The protein intensities were normalized by scaling the median protein intensity in each sample to the same values.

For the two-group statistical analysis the statistical testing was performed using *t*-test on transformed protein intensities (hyperbolic arcsine transformation). Significantly differentially abundant proteins had if linear fold-change < 1.5-fold and the *p*-value from the t-test > 0.05. The full MaxQuant and all the statistical evaluation were performed within B-Fabric, the FGCZ's Core Facility Information Management System [20].

Full data are available via ProteomeXchange with identifier PXD013222.

2.7. Functional characterization of the whole proteome

Gene ontology categorization for all quantified proteins was conducted using the web-based tool PANTHER to assign single top functions to the proteins of the plasma proteome of cows post-calving in various states of infection. The entirety of the bovine proteome was used as a background list for the quantified proteins.

2.8. Characterization of differentially regulated proteins

Four 2-group comparisons were performed to detect differential abundant proteins in peripheral blood of animals with or without endometritis. For individual comparisons, gene ontology term summation was achieved by entering all gene ontology terms associated with one treatment into the online tools ReviGO; pie charts for GO characterization of whole plasma were produced using the online tool PANTHER. The most promising biomarkers were taken from the ANOVA non-directional test with zero imputed values to ensure the best single marker follow-up.

2.9. Statistical analysis

For the comparison of two conditions the statistical testing was performed using a *t*-test on transformed protein intensities (hyperbolic arcsine transformation). Significantly differentially abundant proteins are accepted if the linear fold-change > 1.5-fold and the *p*-value from the t-test < 0.05. The multigroup four-way ANOVA analysis was conducted to check for non-directional significance. Also here, the ANOVA was performed on the hyperbolic arcsine transformed protein intensities. The full MaxQuant and all the statistical evaluation were performed within B-Fabric, the FGCZ's Core Facility Information Management System [20].

3. Results

Mass spectrometry quantification and additional information on all proteins are available in Supplementary materials Table S1 (protein table) and S2 (box-plot quantification for all identified proteins. Species restricted (*Bos taurus*) search of the generated spectra against the UniProt database resulted in the quantification of a total of 181 proteins in the plasma of cattle with varying levels of inflammation. Only proteins identified with at least 2 peptides were considered for quantification. GO slims, utilizing PANTHER, resulted in a broad characterization of proteins present in the plasma proteome of postpartum cows regardless of endometritis condition. Fig. 1 summarizes the major biological processes and molecular functions of quantified proteins. Plasma samples collected at 7 DPP display significantly more proteins associated with immune response systems such as 4 binding protein alpha and mannose binding lectin 2 in the group of cows with cytological endometritis. Proteins associated with complement component complement activation and immune responses mediated by circulating immunoglobulin were also in greater concentrations, as well as proteins associated with proteolysis such as coagulation factor II, thrombin and serpin family A member 1. The plasma results compared to the entire bovine proteome as a background list displayed large numbers of secreted protein complexes and extracellular exosomes. The molecular function of these proteins showed that plasma has more enzyme regulation activity, lipid binding and ion binding than the background of the whole susbet of proteomic terms associated with the cattle genome from the gene ontology consortium.

Five to ten differentially abundant proteins per comparison were found. The summary of differential protein information for the comparisons is found in Table 2. Each comparison is detailed below.

3.1. Cytological endometritis vs healthy (HL/LL vs LH/HH) at 7 DPP

When comparing between healthy animals and those with endometritis, five proteins were upregulated ($P < .05$; Table 2 and Fig. 2). The abundant proteins detected included coagulation factor X1 (F11), glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), Fructose-biphosphate aldolase (ALDOA) and two uncharacterised proteins that could not be BLASTed to any orthologues. Some biological processes observed in the differentially abundant proteins in animals with endometritis (HH/LH) include inflammatory responses, renal

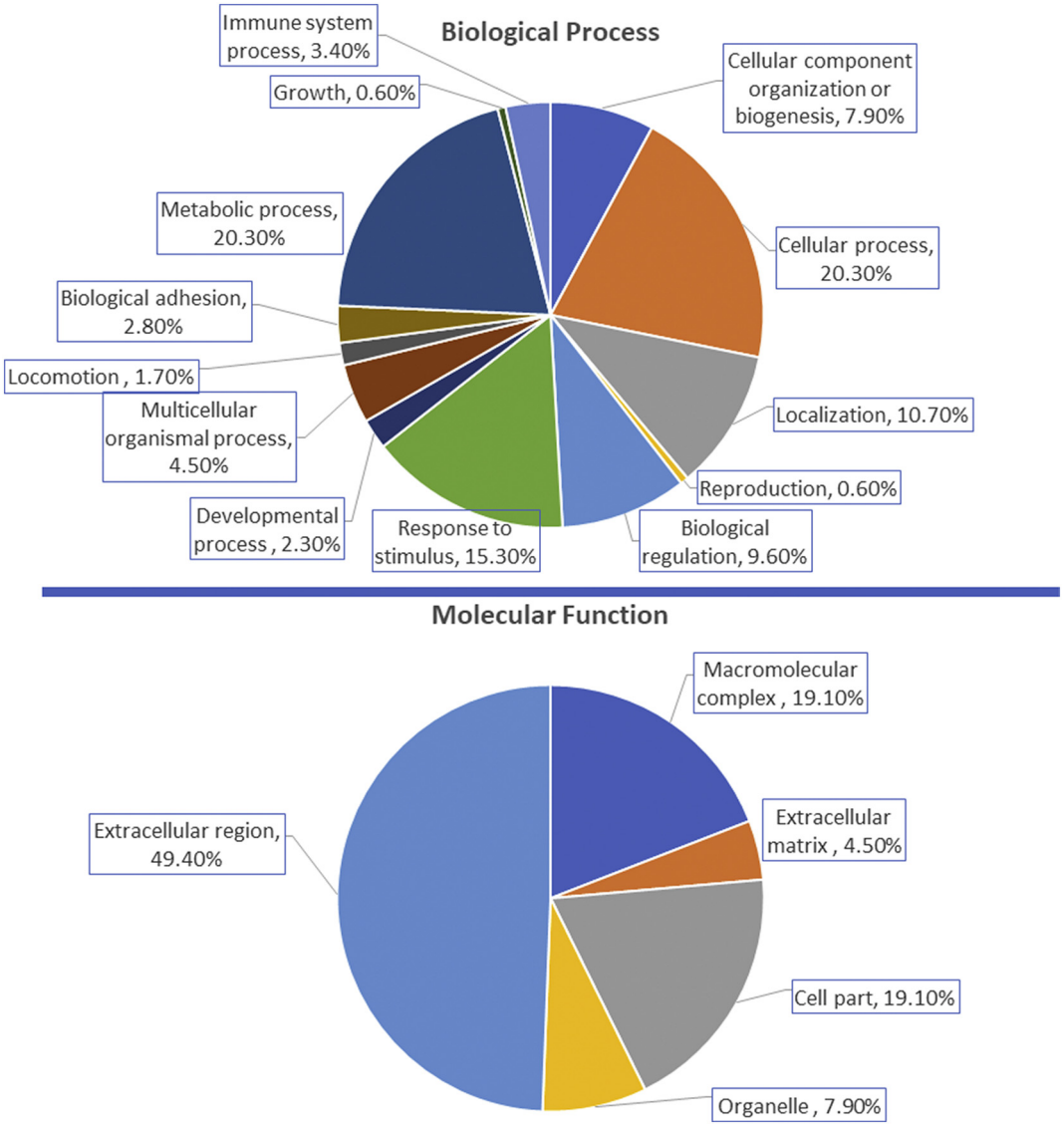

Fig. 1. Biological Process and Molecular Function GO slims produced by the PANTHER online tool for the complete, quantified proteome of bovine plasma utilizing the entire bovine uniprot database versus quantified proteins. This broad classification of the plasma proteome is regardless of endometritis status.

system processes, epithelial cell differentiation, protein localization, postive regulation of cholesterol esterification and lamellipodium assembly. The biologial processes observed in healthy (LL/HL) animals included primarily complement activation.

3.2. High endometrial inflammation (HL vs HH) at 7 DPP

When comparing protein changes in the plasma of animals which initially had prominent endometrial inflammation but regressed to healthy, to those that maintained a prominent cellular efflux from the uterus from 7 to 21 DPP, the animals with persistent endometrial inflammation, displayed an abundance of 6 proteins. These proteins were associated with phagocytosis and the innate immune response, including two uncharacterized proteins previously known only from RNA transcriptome results. However, 4 proteins were upregulated in HL animals. These are secreted phosphoprotein 24 (Spp-24), serum amyloid P component (SAP), protein HP-20 homolog and GlyCAM-1. Two uncharacterized proteins were upregulated in animals with persistent endometerial inflammation.

3.3. Minimal inflammation (LL vs LH) at 7 DPP

When comparing those that consistently maintained a low efflux of inflammatory cells from the endometrium (LL) to those that originally had low levels but developed endometritis (LH), 12 proteins were differentially abundant between the two groups. Nine proteins were abundantly produced in LL animals compared to LH animals, whilst 3 proteins were increased in LH animals relative to LL animals. Proteins abundant in LL animals include proteins such as complement C5a anaphylatoxin (C5), coagulation factor XI (F11), complement C8 beta chain (C8B), peroxiredoxin 2 (PRDX2) and 4 uncharacterised proteins that could not be BLASTed to any orthologues. Proteins abundant in LH animals include kininogen-2 (KNG2) and inter-alpha-trypsin inhibitor heavy chain H3 (ITI-HC3). LL animals demonstrated apoptosis regulatory activity, striated muscle contraction, fructose metabolism, immoglobulin production, plasminogen activation and phagocytosis engulfment such as the same previously mentioned unreviewed and uncharacterized protein with the ID G5E513. LH animals produced proteins associated with the notch signlaing pathway (implicated in blood vessel homeostasis) and vasodilation.

Table 2
Proteins that are differentially abundant between animals based on fold change and *p*-value are listed below. The separate comparisons (column 1) are shown together with the number of animals (column 2). The number of animals (measured/missing are shown in column 3) gether with the associated pValue (column 4) and fold change (column 5). SwissProt accession numbers (Protein ID, column 6) are given for each protein. Shorthand two letters denoted 7 and 21 DPP; L = low neutrophils and H = high (i.e.: HH = high at 7 and 21 DPP, LH means low at 7 DPP and high at 21 DPP).

Comparison	Number of samples	Measured | |Missing values				pValue	Fold Change (Log2)	Protein ID	Protein name
		LL	LH	HL	HH				
HL/LL vs LH/HH		5|0	5|0	5|0	5|0	0.004	0.79	P80195	Glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1)
		5|0	5|4	5|3	4|1	0.027	1.21	A6QLL8	Fructose-bisphosphate aldolase
	20	4|3	5|2	5|1	5|2	0.024	0.99	F1MH40	Uncharacterized protein
		5|0	5|0	5|0	5|0	0.032	1.32	F1MLW8	Uncharacterized protein
		4|1	5|3	5|3	5|3	0.003	1.14	F1MUT4	Coagulation factor XI
LL vs LH		4|1	3|2	–	–	0.03	−2.96	G3X6K8	Haptoglobin
		2|3	3|2	–	–	0.006	−0.84	P01045	Kininogen-2
		5|0	5|0	–	–	0.008	−0.74	P56652	Inter-alpha-trypsin inhibitor heavy chain H3
		5|0	5|0	–	–	0.04	0.73	F1 N514	Uncharacterized protein
	10	5|0	5|0	–	–	0.03	0.75	F1 N102	Complement C8 beta chain
		4|1	5|0	–	–	0.04	1.28	G3MWT1	Uncharacterized protein
		3|2	3|2	–	–	0.04	1.3	Q9BGI3	Peroxiredoxin-2
		3|2	2|3	–	–	0.0006	1.8	F1MH40	Uncharacterized protein
		4|1	3|2	–	–	0.0008	2.15	F1MY85	Complement C5a anaphylatoxin
		5|0	5|0	–	–	0.005	2.26	G5E513	Uncharacterized protein
HL vs HH		–	–	5|0	5|0	0.03	−1.38	G5E513	Uncharacterized protein
		–	–	5|0	5|0	0.03	−0.75	G5E604	Uncharacterized protein
	10	–	–	5|0	5|0	0.05	0.7	Q2KIT0	Protein HP-20 homolog
		–	–	5|0	5|0	0.05	0.99	P80195	Glycosylation-dependent cell adhesion molecule 1
		–	–	5|0	5|0	0.04	1.73	Q3T004	Serum amyloid P-component
		–	–	4|1	5|0	0.03	1.76	Q27967	Secreted phosphoprotein 24

4. Discussion

Despite advances in nutrition, husbandry and veterinary care, endometritis continues to detrimentally affect the dairy industry. Knowing the differentially abundant plasma proteins caused by endometrial inflammation would allow the identification of dairy cows succumbing to endometritis. Thus, at risk animals detected as early as 7 days post-calving can therefore receive timely intervention. Although a number of studies have begun to characterise the uterine transcriptome [12], few have focused on the bovine plasma proteome postpartum. More recently, the endometrial and plasma proteome of animals with or without endometritis was investigated at 21–30 DPP [21]. They identified CD14, MMP3, and MMP9 proteins with important functions in the cross-talk of pathways related to extracellular proteolysis [18], and concluded that these proteins may promote inflammation and hence endometritis. A recent study showed that many pathways involved in a wide range of immune functions are affected by LPS at the mRNAs and proteins level [22]. LPS was found to induce proinflammation and down-regulate galectin-1 [22]. Here we identify proteins differentiated in healthy and animals with clinical endometritis. Whereas cytological endometritis resulted in an increase in the expression of proteins associated with muscle contraction and homeostasis, in vitro studies showed that LPS activated proteins involved in energy metabolosim and immune response [22], both of which play key roles in the pathogenesis of endometritis [23]. It has been shown that collectin-43 precursor, deoxyribonuclease-I (DNase-I), and MHC class I heavy chain (MHC-Ih) were up-regulated in the endometrium of cattle with endometritis [24]. Here MHC-1 was upregulated in the plasma of catttle thay eventually developed endometritis. These results, and our findings here of an upregulation in uncharacterized protein of interest, G5E513 (an immunoglobulin M-like protein), is found to be significant in both the two-way comparisons as well the ANOVA test and is consistently higher in animals suffering from endometritis. It was BLASTed to immunoglobulin-M or is an immunoglobulin M-like protein. Aforementioned, we summarize the properties of identified proteins in the plasma of endometritic animals (Table 2).

Severity of inflammation (H-high) 21 DPP presented clear differential abundance of protein associated with onset of inflammation. Predictably, this included several proteins with immune function. Protein GlyCAM-1 binds to the L-selectin receptor during inflammation, thereby promoting leukocytes recruitment to sites of inflammation [25]. GlyCAM-1 is a protein that likely acts as an inhibitory modulator of cell adhesion [26]. When animals suffer inflammation, such proteins are immediately synthesised. However, little information is currently available on the alterations of plasma proteins concentrations during endometritis. Here, the plasma of healthy animals showed expression of complement C5a anaphylatoxin (C5), which plays a role in compliment activation [27]. The increase in these proteins is part of the inflammatory process. In vitro, bovine endometrial epithelial cells stimulated with LPS differentially express proteins associated with cell proliferation and apoptosis, transcription, destabilization of cell structure, oxidative stress, regulation of histones, allergy and general cell metabolism pathways [22]. Similarly in this study, serum amyloid P (SAA), an acute phase protein (APP) which binds to pathogens, was also increased in healthy animals. This is in contrast to previous reports where serum amyloid a (SAA) and haptoglobin (Hp) increase in circulation in 7 DPP in cattle predisposed to uterine disease [28]. However, animals with low inflammation intially (LH) that later developed endometritis had elevated plasma levels of Hp [12]. Even though the complement system plays an important role in the activation of inflammation, two complement proteins were increased in LL animals. These are C5a, a key mediator of inflammation, and C8b, a component of C8 – part of the membrane attack complex. They function to create pores in bacterial cell membranes, during bacteria killing. Low levels of these proteins in animals that suffer endometritis point to inadequate antibacterial defence mechanisms or delayed pathogen clearance resulting in uterine disease, both of which have negative consequences for uterine health.

This study draws proteomic comparisons between animals with, and

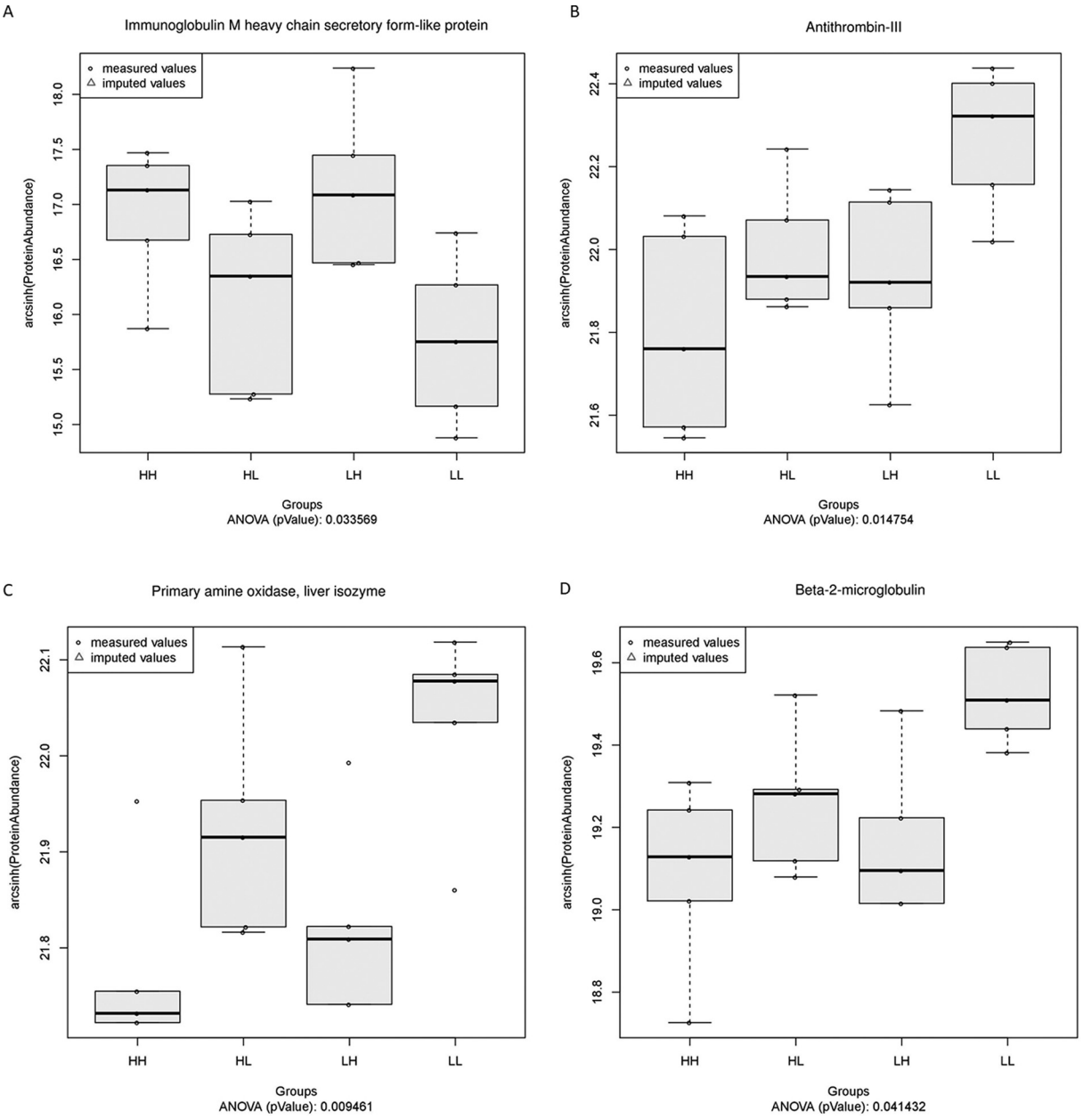

Fig. 2. The multigroup analysis resulted in boxplots for all samples and all identified proteins. The ten significant proteins from the ANOVA test were screened for abundance patterns that showed high abundances in animals that were positive for endometritis at the end of the period (plot A) but low in others, high abundance in animals that were healthy throughout but low in others (B and D) or high abundance in animals that were healthy at the end regardless of starting condition (C) versus others. All proteins selected for biomarker followup have no imputes amongst the measured proteins.

without endometritis to suggest potential prognostic indicators of uterine disease, and identifies several proteins of interest. Four proteins from the list of 10 significant proteins demonstrating relative abundances merit further study as potential markers (their relative abundances between comparisons are shown in Fig. 2) due to their abundance patterns (that is, only higher in abundance in healthy or endometritis animals depending on the protein). The other six proteins do not show such abundance patterns, and may not serve as accurate biomarkers of endometritis. The most promising single marker to come from the study is the Uncharacterized Protein G5E513 (Fig. 2A), a protein which matches well via FASTA sequence to the recently described bovine version of Immunoglobulin M Heavy Chain Secretory form. Immunoglobulin M is one of the first antibodies to appear in response to antigen in most vertebrates it is found in and is a large, prominent molecule that is well described in mammals [29]. It's higher in abundance in both sets of animals that are over the 18% threshold at the end of the measurement period (LH and HH) versus those that are not (LL and HL). It's also shown up as significant within several directed comparisons (see Table 2 for Uncharacterized protein ID G5E513). It's abundance pattern shows great promise as a part of a selection index or even a single, measurable biomarker available to dairy cattle farmers if refined. Antithrombin-III is found to be relatively higher in animals that never displayed a neutrophil count over 18% (LL) and were thus healthy throughout the measurement period. It inactivates several proteins of the coagulation system [30]. It is worth noting that it shares an abundance pattern with beta-2-microglobulin, which is also relatively lower in abundance in animals that never succumed to endometritis (Fig. 2D). Beta-2-microglobulin is a key regulator of hepcidin which in turn regulates iron transport; it's relation to the immune system has also been studied extensively since the 1960s as lower levels

of beta-2-microglobulin are associated with immuno-comrpomise in human patients, such as those with lymphoma [31,32]. Taken together, both antithrombin-III and beta-2-microglobulin's distincitve patterns of abundance in animals that never succumbed to endometritis suggests that low counts of either or both protein might signal endometritis. As such, only healthy animals during the experiment demonstrate higher abundance. Other proteins significantly increased in endometria of cattle with endometritis, but not upregulated here include desmin and alpha-actin −2 invloved in cellular apotosis [26]. These are likely to be highly secreted during endometritis due to high tissue inflammation [33]. Primary amine oxidase, liver-isoszyme is low in animals that tested positive for endometritis at the end of the study period but is lower in animals that either never succumbed to OR cleared the infection (Fig. 2C). Its function is not well understood but it is known to be involved in inflammation; it's key for leukocyte migration to sites of inflammation [34]. Its anti-inflammatory properties make it an unsurprising find in animals that are clearing infection and inversely its relatively lower abundance in infected animals suggests that the anti-inflammatory proteins and enzymes associated with this protein and its pathways are perhaps lower in abundance.

The uncharacterized immunglobulin M-like protein, G5E513 in particular shows great promise as a single biomarker of endometritis. It's function as an early responder of the innate immune system suggests that this protein will be present early and in high concentrations in animals that may succumb to clinical endometritis.

5. Conclusions

This study identified the uncharacterized immunoglobulin M-like protein G5E513 abundanltly expressed in the plasma of dairy cattle suffering from endometritis. This protein merits further investigation as potential biomarkers of endometrtitis because it may shed light on proteins whose deficiency is associated with immuno-compromise. The study of G5E513 and phosphoprotein 24 (Spp-24), serum amyloid P component (SAP), protein HP-20 homolog and GlyCAM-1 found to be statistically significant here will likely yield additional useable information for dairy producers to more effectively diagnose early postpartum cattle at risk of developing endometritis.

Funding

Funding to AC was provided by the Center for Integrative Mammalian Research, Ross University School of Veterinary Medicine. Funding to KGM was provided by the Irish Department of Agriculture, Food and the Marine (Stimulus 13/S/472).

Declaration of Competing Interest

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2019.103421.

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